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Interactions of Gephyrotoxin with the Acetylcholine Receptor-Ionic Channel Complex

II. Enhancement of Desensitization

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SUMMARY

The actions of the tricyclic alkaloid gephyrotoxin (GyTX) on the extrajunctional and junctional acetylcholine (ACh) sensitivity and desensitization were studied in the chronically denervated rat soleus muscle and cutaneous pectoris muscle of the frog. At low concentrations, GyTX greatly depressed the extrajunctional ACh sensitivity of the chronically denervated soleus muscles. In addition, GyTX produced a strong inhibition of junctional end-plate potentials evoked by ACh. Junctional and extrajunctional desensitizations induced by microiontophoretically applied ACh were greatly enhanced by the alkaloid in a frequency-dependent manner. These effects were readily reversible. The interaction of GyTX with binding sites on the acetylcholine receptor-channel (AChR) complex was studied on electroplax membranes from *Torpedo californica*. GyTX binds to the AChR complex at a site distinct from the ACh binding site, as revealed by its lack of inhibition of [^{125}I]α-bungarotoxin ([^{125}I]BGT) binding. On the other hand, GyTX at a concentration range between 1 μM and 100 μM significantly increased the potency of the agonist carbamylcholine as an antagonist of binding of [^{125}I]BGT. At low micromolar concentrations, GyTX inhibited the binding of [^{3}H]perhydrohistrionicotoxin and [^{3}H]phencyclidine to sites associated with the ionic channel of the AChR complex. The affinity of GyTX for these sites was increased 3- to 5-fold by carbamylcholine. Results of electrophysiological and binding studies indicate that GyTX not only blocks the open channel of the AChR but also enhances desensitization of the AChR complex by increasing receptor affinity for agonists.

INTRODUCTION

Desensitization in neuromuscular preparations is apparently related to a modification of the conformational state of the AChR[6] complex which occurs through an interaction with acetylcholine or other agonist. This conformational change results in a nonconducting state. As reported by Thesleff (1), muscle depolarization gradually diminishes in response to a long-lasting application of agonist. Receptor activation by the agonist then does not occur until complete relaxation of the AChR complex to the resting state is achieved. This relaxation time is related to the concentration of the agonist and to the time required for dissociation of the agonist from its binding site, thereby allowing the AChR complex to attain its initial conformational state. Certain agents have been shown to facilitate the formation of a desensitized state of the AChR complex, not by a direct effect but by increasing the affinity of the agonist for the receptor site. As a consequence of increased binding of the agonist, desensitization will occur in the presence of the agent, at concentrations of the agonist not sufficient

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[6] The abbreviations used are: ACh, acetylcholine; AChR, acetylcholine receptor-ion channel complex; GyTX, gephyrotoxin; HTX, histrionicotoxin; H_{12}-HTX, perhydrohistrionicotoxin; BGT, α-bungarotoxin; PCP, phencyclidine.

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to induce desensitization if applied alone. Among these agents are PCP (2), meproadifen (3, 4), phenothiazines (5), HTX (6–9), and lidocaine (10).

The present electrophysiological and biochemical studies with frog and rat neuromuscular synapses and with *Torpedo* electric organ suggest that GyTX, an alkaloid from a Colombian poison frog (11), in addition to blocking the open conformation of the ionic channel of the AChR complex, causes a marked increase in affinity of the agonist for the ACh receptor and therefore induces desensitization.

MATERIALS AND METHODS

Electrophysiological techniques. The extrajunctional sensitivity to iontophoretically applied ACh was studied in denervated (11–15 days) rat soleus muscle using standard published techniques (12). High-resistance micropipettes (150–300 Mohm) filled with 4 M ACh were used to induce ACh potentials at 1, 2, and 5 Hz, with pulse duration ranging from 0.5 to 2 msec. Throughout the experiment, muscles were perfused with the following solution (millimolar): NaCl, 135.0; KCl, 5.0; $CaCl_2$, 2.0; $MgCl_2$, 1.0; $NaHCO_3$, 15.0; NaH_2PO_4, 1.0; and glucose, 11.0. The perfusing solution was continuously bubbled with 95% O_2/5% CO_2 and had a pH around 7.2–7.3.

Junctional ACh sensitivity was studied in intact frog cutaneous pectoris muscles using the conventional double-barreled pipette technique of Katz and Thesleff (13). Repetitive short-duration pulses (50 μsec) were applied every second, superimposed on a long-duration, steady ACh pulse (30 sec). Muscles were perfused throughout the experiment by normal frog Ringer's solution as described in the previous paper (14).

The results of junctional and extrajunctional ACh sensitivity are expressed as the ACh-induced depolarization (mV) per charge delivered to the microiontophoretic pipette (nC) (15).

Biochemical tissue preparation. Torpedo californica electric organs were purchased from Pacific Biomarine and stored at −70° for up to 3 months before use. Membranes were homogenized with 20 volumes of 50 mM Tris-HCl (pH 7.4) containing 1 mM phenylmethylsulfonylfluoride to inhibit proteolytic enzymes in a Waring blender at the highest speed for 30 sec. After filtering through four layers of cheesecloth, the suspension was centrifuged at 15,000 × *g* for 20 min. The pellets were resuspended in buffer and used without further treatment. Protein content was determined by a modification of the method of Lowry *et al.* (16).

Binding assays. Ion channel binding was measured using [^{3}H]PCP ([*piperidyl*-3,4-^{3}H(N)]-PCP, 48 Ci/mmole; New England Nuclear Corporation, Boston, Mass.) and [^{3}H]H_{12}-HTX, about 54.5 Ci/mmole (prepared by New England Nuclear Corporation by reduction of octahydrohistrionicotoxin) as probes. To measure binding, an aliquot of the membrane suspension (40–60 μg of protein) was added to an incubation medium containing 3 nM [^{3}H]PCP or 2 nM [^{3}H]H_{12}-HTX and Tris buffer in a final volume of 1 ml. Parallel experiments were run in the presence or absence of 1 μM carbamylcholine, which, along with most receptor agonists, profoundly influences ion channel binding properties (17). Nonspecific binding was determined in the presence of 100 μM amantadine, a potent channel blocker (18). After incubation for 60 min at room temperature, the suspension was filtered through Whatman GF/B glass-fiber filters which had been wetted with a 1% organosilane preparation (Prosil 28; PCR Research Chemicals, Gainesville, Fla.) to reduce nonspecific binding to the filters. The filters were washed twice with 5 ml of Tris buffer and removed for determination of their radioactivity content by liquid scintillation counting as described elsewhere (19). The $t_{1/2}$ for dissociation of [^{3}H]H_{12}-HTX or [^{3}H]PCP was about 2 min. The time of washing and filtration was less than 10 sec, during which time less than 5% of the specifically bound channel probe was dissociated.

Nicotinic receptor binding was measured using [^{125}I]α-bungarotoxin. [^{125}I]BGT (New England Nuclear Corporation). To measure [^{125}I]BGT binding, a filtration assay was used. *Torpedo* membranes (10–15 μg of protein) were incubated with the appropriate ligands (e.g., GyTX or carbamylcholine) for 20 min in 1 ml of Tris-HCl (pH 7.4) before initiation of the binding reaction by addition of 5 nM [^{125}I]BGT. After 20 min, the reaction was quenched by addition of 0.5 ml of methylated bovine serum albumin (10 mg/ml) (Sigma Chemical Company, St. Louis, Mo.). The suspensions were then filtered through Whatman GF/B filters which had been soaked in the albumin solution. The radioactivity content of the filters was determined by gamma scintillation counting. Nonspecific binding was determined by including 100 μM *d*-tubocurarine in a parallel set of incubations.

GyTX, isolated from *Dendrobates histrionicus*, was used from a concentrated stock solution (10 mM in 95% ethanol) kept at 4°. The natural compound is the *l*-enantiomer. A sample of synthetic *d*-GyTX was kindly provided by Prof. Y. Kishi (Harvard University). Unless otherwise noted, natural GyTX was used in all studies. We are indebted to Dr. T. Tokuyama (Osaka City University) for additional supplies of GyTX.

RESULTS

ACh sensitivity of intact and chronically denervated muscles in the presence of GyTX. The ACh sensitivity of surface fibers of chronically denervated rat soleus muscle was studied by measuring the amount of depolarization induced by a given dose of microiontophoretically applied ACh. The effect of GyTX in this preparation was dependent upon the concentration of alkaloid, duration of exposure, and frequency of stimulation. Within 30 min of exposure to GyTX, 20 and 30 μM, the ACh sensitivity of the denervated rat soleus muscle was reduced to 28.1 ± 3.8 mV/nC and 40.0 ± 4.0 mV/nC, respectively, from a control value of 514.0 ± 44.0 mV/nC (13 fibers, 4 muscles). Under control conditions, repetitive application of ACh in series of 10 pulses, at 1, 2, and 5 Hz, did not alter significantly the amplitude of ACh potentials (Fig. 1). However, in the presence of 20 μM GyTX, the amplitude of the last ACh potential in a train of 10 pulses, elicited at frequencies of 1, 2, and 5 Hz, was

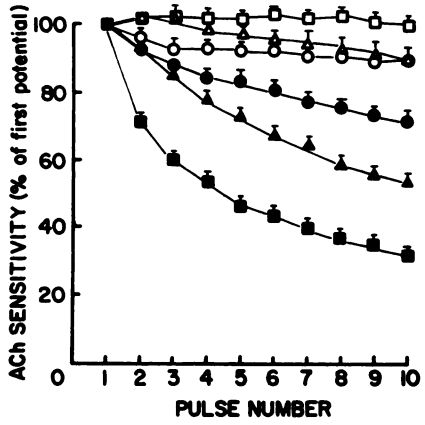

FIG. 1. *Acetylcholine sensitivity of denervated rat soleus muscle: effect of GyTX and frequency of stimulation on the microiontophoretically induced ACh potential*

ACh sensitivity is expressed as a percentage of the first ACh potential induced in a train of 10 stimuli at 1 Hz (○, ●), 2 Hz (△, ▲), and 5 Hz (□, ■). *Open symbols* refer to the control condition and *closed symbols* after exposure of the preparation to 20 μM GyTX for 30 min. Each point represents the mean ± standard error of the mean of 15 measurements in 5 different fibers.

reduced by 28%, 46% and 68% in relation to control, respectively. In other words, increasing the frequency of stimulation, and therefore having more ACh available at the AChR complex, leads to a more dramatic decrease in the ACh potentials. Once one train was elicited, at any frequency, the last ACh potential was maximally depressed and other trains elicited after 15 sec of the first failed to induce any further decrement in that ACh potential. No more than 5 sec were required for complete recovery of the first ACh potential at any of the frequencies studied. Although we are reporting data obtained after 30 min of exposure to GyTX, it is worth mentioning that longer exposures progressively decreased the ACh sensitivity of the denervated rat soleus muscle.

The effect of GyTX was also studied on the onset of desensitization at junctional regions of the innervated frog cutaneous muscle. In this type of experiment, short duration (50 μsec) ACh pulses are delivered, at a rate of 1 sec^{-1}, through one barrel of a double-barreled pipette. The other barrel delivers a steady 30-sec duration ACh pulse. The decrement observed in the ACh potentials induced by the short pulses with time is taken as a measure of desensitization (13). Figure 2 shows a representative experiment. Under control conditions, a steady ACh-induced depolarization of 3–5 mV caused only a small desensitization; i.e., the amplitude of the last brief ACh potential, concomitantly induced with the steady depolarization, was reduced to about 50–60% of the first one. Full recovery of those brief potentials was observed after interruption of the long-lasting ACh pulse for approximately 20 sec (Fig. 2A and B, *upper panels*). In the presence of GyTX, however, the onset and extent of depolarization became dependent on the concentration of the toxin and period of incubation. For example, incubating the muscle in the presence of 10 μM GyTX for 10 and 20 min decreased the last short ACh potential to 20% and 5% of the control value, respectively (Fig. 2A, *middle* and *lower panels*). Stopping the steady ACh pulse caused the brief potentials to recover to 80% of the control value in about 1 min.

Complete blockade of the brief response was accomplished after a 40-min incubation with 10 μM GyTX, but it required only 20 min if the concentration was 20 μM (Fig. 3). In fact, incubation with 20 μM GyTX for 5 min almost completely abolished the brief response (Fig. 2B, *middle panel*). Stopping the steady ACh pulse for 1 min was enough to bring the brief response, under this condition, to about 70% of the initial values. Washing the preparation for 60 min with GyTX-free Ringer's solution fully restored the responses to control conditions (Fig. 2B, *lower panel*). GyTX not only depresses the brief ACh response, but has an effect on the steady depolarization as well.

Binding studies in Torpedo electroplax membranes. GyTX inhibited the binding of both [^{3}H]H_{12}-HTX and [^{3}H]PCP to the AChR complex in membranes isolated

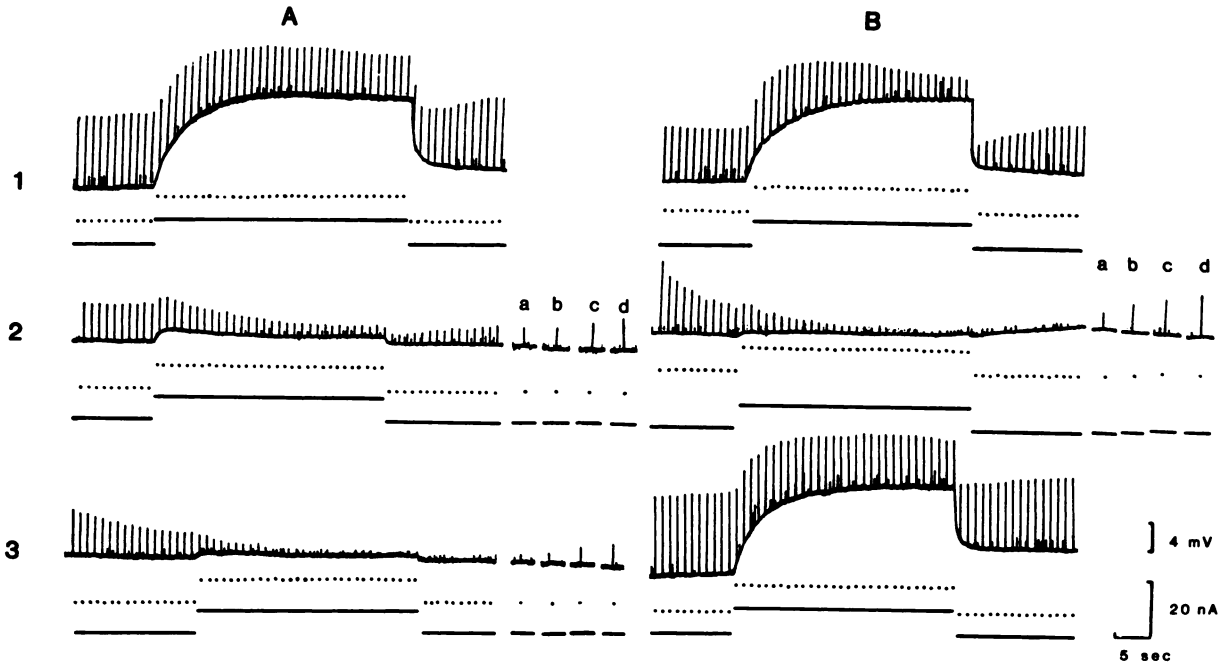

FIG. 2. *Polygraphic records of microiontophoretic ACh-induced potentials in frog cutaneous pectoris muscle*
A double-barreled micropipette was used to deliver both a steady long-lasting pulse (30-sec duration, indicated by the *heavy line* under each trace) and a short pulse (50-μsec duration, indicated by the *dotted line* under each trace, which was retouched in order to indicate the point). The uppermost traces are controls. In A, the muscle was exposed to 10 μM GyTX for 10 min (*middle trace*) and 20 min (*lower trace*). In B, the muscle was exposed to 20 μM GyTX for 5 min (*middle trace*) and then washed for 60 min (*lower trace*). *a*, *b*, *c*, and *d* are ACh potentials evoked 15, 30, 45, and 60 sec, respectively, after stopping the long-lasting ACh pulse. Cell resting potential in A was −75 mV and in B −80 mV. Vertical bars are calibrations for the extent of cell depolarization, in millivolts, and for current delivered through the microiontophoretic pipette in nanoamperes. The horizontal bar shows time in seconds.

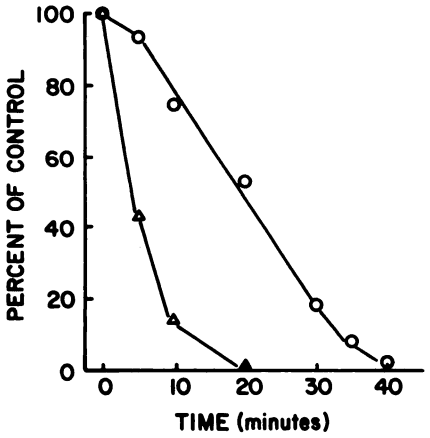

FIG. 3. *Time course for the onset of effects of GyTX, 10 μM (○) and 20 μM (△), on the amplitude of the first ACh potential elicited by a short-duration ACh pulse, superimposed on a long-lasting ACh pulse*

The first ACh-evoked potential is expressed as a percentage of the same response recorded under control conditions, i.e., in the absence of GyTX.

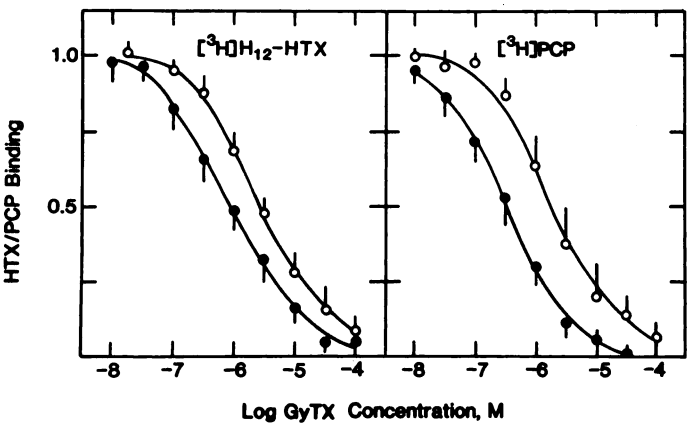

FIG. 4. *GyTX inhibition of [³H]PCP and [³H]H_{12}-HTX binding to the AChR complex in membranes prepared from Torpedo californica electroplax*

Inhibition of specific [³H]H_{12}-HTX (*left*) and [³H]PCP (*right*) binding by GyTX was measured in the absence (○) and presence (●) of 1 μM carbamylcholine. Binding is expressed as fraction of total specific binding measured in the absence of gephyrotoxin. Each point and bar represents the mean ± standard deviation from four determinations.

from *Torpedo* electric organ (Fig. 4; Table 1). GyTX had a somewhat higher affinity versus binding of [³H]PCP (IC_{50} = 1.8 μM) than versus binding of [³H]H_{12}-HTX (IC_{50} = 2.8 μM). The affinity of GyTX was increased 3- to 5-fold when carbamylcholine was included in the incubation medium. This allosteric interaction between "receptor" and "channel" binding sites is seen with most channel ligands. The inhibition of binding could be reversed by washing the membranes by centrifugation. Little stereospecificity was associated with the binding insofar as the naturally occurring *l*-GyTX was as potent as synthetic *d*-GyTX in inhibiting [³H]H_{12}-HTX and [³H]PCP binding to channel sites (Table 1).

In contrast, GyTX had minimal affinity for the nicotinic receptor (i.e., ACh binding site) in *Torpedo* electric organ. At concentrations up to 100 μM, GyTX did not inhibit the binding of 5 nM [¹²⁵I]BGT to *Torpedo* membranes.

TABLE 1

Inhibition by GyTX of [³H]PCP and [³H]H_{12}-HTX binding to the AChR complex in membranes from Torpedo electroplax

The apparent inhibition constants (K_i) for *l*- and *d*-GyTX inhibition of [³H]H_{12}-HTX and [³H]PCP binding to *Torpedo* receptor-channel complexes are indicated. Inhibition constants were calculated from GyTX concentrations which inhibited by 50% the specific binding of 2 nM [³H]H_{12}HTX and 3 nM [³H]PCP according to the relationship, $K_i = IC_{50}/(1 + L/D)$, where L is the concentration of radiolabeled channel probe and D is the channel probe dissociation constant. [³H]H_{12}HTX dissociation constants were 0.135 and 0.089 μM in the absence and presence of 1 μM carbamylcholine (Carb), respectively. [³H]PCP dissociation constants were 0.54 and 0.14 μM in the absence and presence of carbamylcholine. In GyTX/[³H]H_{12}TX and GyTX/[³H]PCP competition experiments, the channel probes were present at only a small fraction of their dissociation constant value (i.e., $L \ll D$). Therefore, only a small adjustment was necessary when calculating inhibition constants from the measured IC_{50}. It should be noted that this analysis assumes an interaction with a single population of noninteracting binding sites. The means ± standard deviation from four determinations are shown.

Compound	K_i (μM)			
	[³H]H_{12}-HTX		[³H]PCP	
	Control	1 μM Carb	Control	1 μM Carb
l-GyTX (natural)	2.8 ± 1.2	0.87 ± 0.09	1.8 ± 0.7	0.36 ± 0.06
d-GyTX (synthetic)	2.0 ± 1.0	0.78 ± 0.08	1.5 ± 0.5	0.37 ± 0.07

Prolonged exposure of nicotinic receptors to receptor agonists induces a "desensitized" state of the receptor-channel complex that is characterized by a higher affinity of the receptor for agonists (20, 21). A number of ionic channel blockers appear to select for an inactive conformation of the AChR complex which is characterized by an increased affinity for agonists, and which may be identical with the desensitized AChR complex. GyTX enhanced the ability of carbamylcholine, a stable receptor agonist, to inhibit the binding of the receptor agonist [¹²⁵I]BGT (Fig. 5). This increase was observed at GyTX concentrations above 1 μM and was maximal at 100 μM, a concentration range corresponding to 50–100% occupation of channel binding sites.

DISCUSSION

A number of compounds have been shown to interact with the AChR complex, interrupting neuromuscular transmission, by two essentially distinct mechanisms: (a) blocking the ion channel in its open conformation, and/or (b) increasing receptor affinity for the agonist, ultimately leading to desensitization (see, for example, refs. 4, 6, 7, and 22–25).

In the previous paper (14) we have shown that the results obtained with GyTX could be explained by assuming blockade of open channels as a mechanism for the action of this alkaloid. However, the use-dependent effect induced by GyTX can also be taken as indicative of a desensitization process. Even the more pronounced depression in peak end-plate current amplitude at hyperpolarizing voltages could be related to the voltage dependence seen in desensitization studies, whether induced by bath or iontophoretic ACh application (26, 27) or by nerve released transmitter (28). In the present paper we show that GyTX does interact with the AChR

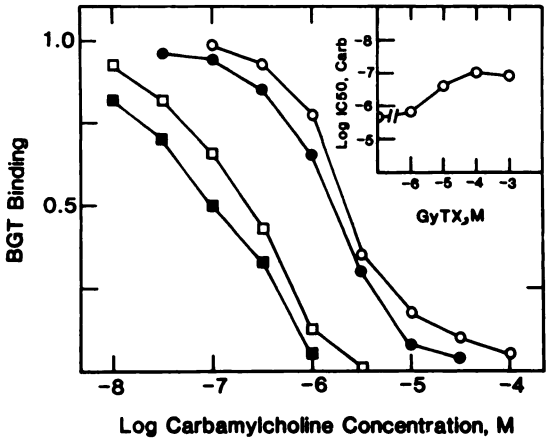

FIG. 5. *Effect of GyTX on carbamylcholine inhibition of [^{125}I]BGT binding to the AChR complex membranes from Torpedo electroplax*

The binding of [^{125}I]BGT was measured in the presence of the indicated concentrations of carbamylcholine. GyTX at 0 (○), 1 (●), 10 (□), and 100 (■) μM was included in the incubation media. GyTX did not affect the level of [^{125}I]BGT binding in the absence of carbamylcholine. The GyTX-induced increases in carbamylcholine affinity for the [^{125}I]BGT binding site are summarized in the *inset*: The log of the carbamylcholine concentration at which [^{125}]BGT binding was inhibited by 50% (*log IC_{50}, Carb*) is plotted as a function of the GyTX concentration. Each binding point represents the mean from three determinations. Each IC_{50} value is the mean from three separate determinations which differed by less than 40%.

complex so as to increase receptor affinity for agonists and cause desensitization. Such an effect is seen in Fig. 1, where an increase in frequency of the microiontophoretically applied ACh, in denervated rat soleus muscle leads to a sharp decrease in ACh sensitivity. This decrease in sensitivity is complete in a few seconds, dependent on the frequency of ACh application, and is completely reversible upon washing out GyTX. The fast time course and recovery are in marked contrast to the effect of GyTX on peak end-plate current amplitude and decay time constant, where a progressive decrease was seen for hours (14). The results in Figs. 2 and 3 lend further support to the idea that GyTX increases receptor affinity for the agonist; i.e., desensitization in the frog cutaneous pectoris was greatly enhanced in the presence of GyTX. Results similar to these have been reported for other drugs known to interact with both the open and closed configuration of the ACh ion channel, such as HTX (6, 7, 23) and PCP (2) and for drugs acting only on the closed-channel conformation of the AChR, such as meproadifen (3, 4) and phenothiazines (5). Furthermore, as mentioned in the previous paper (14), this effect of GyTX leading to receptor desensitization and an increase in affinity of ACh to its binding sites is not related to the open-channel blockade produced by the toxin. Several lines of evidence support these conclusions: (a) open blockade occurred earlier than desensitization[7] (29); and (b) agents such as meproadifen, which induces a marked degree of receptor desensitization (3, 4), do so without affecting either channel life time or channel conductance (30).

In biochemical studies, GyTX inhibited binding of [^{3}H]PCP and of [^{3}H]H_{12}-HTX to *Torpedo* electroplax membranes (Fig. 4). GyTX was more potent as an antagonist versus [^{3}H]PCP than versus [^{3}H]H_{12}-HTX. These binding sites appear to be distinct from the ACh binding site (2, 8). Indeed, GyTX did not inhibit the binding of [^{125}I]BGT. On the other hand, GyTX increases the affinity of the AChR complex for carbamylcholine, a stable agonist (Fig. 5). Drugs that do not induce a considerable desensitization, such as atropine and piperocaine, but do have an effect on single-channel lifetime and decay time constant of the end-plate current (31, 32) bind to the AChR complex with the same affinity, either in the presence or absence of carbamylcholine (33).

Whether GyTX, H_{12}-HTX, and PCP need two binding sites in order to express all of their effects is not clear from our experiments, since it is possible that the site used for open-channel blockade could also be responsible for the increase in affinity for agonists at the receptor, i.e., desensitization. Such mode of action has been proposed for certain local anesthetics (24). However, the rapid onset and reversal of effects of GyTX on desensitization phenomena are in marked contrast to the slow onset and reversal of channel blockade, suggesting the operation of two distinct mechanisms (29).

[7] Y. Aracava and E. X. Albuquerque, unpublished observations.

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